OBSERVATIONS ON THE THERMALLY INDUCED ISOMETRIC TENSION IN COLLAGENOUS FIBERS OF RAT TAIL TENDON WITH REFERENCE TO LATHYRISM

ABSTRACT

The isometric tension which develops in rat tail tendon fibers near the shrinkage temperature was measured and plotted against the time. The resulting curves were studied in various conditions. Two types of deviation from the normal were found: (*a*) accelerated development of tension and (*b*) accelerated relaxation, which is often associated with lower maximal tension.

Pretreatment in sodium chloride and acetic acid solutions caused an acceleration in the development of tension and also a premature relaxation. Storage of the fibers and immersion in urea solution hastened the relaxation.

Samples from lathyritic rats showed an accelerated relaxation. The tensile strength per cross-sectional area of the fiber was decreased.

It is suggested that the first phase, development of tension, depends on the same factors as the thermal or chemical contraction and that the relaxation depends sensitively on the various factors which determine the frictional forces between the subunits.

INTRODUCTION

The original purpose of this work was to study whether the amount of the neutral salt–soluble fraction in collagen influenced the mechanical and thermal properties of the fibers in rat tail tendon. It is known that this fraction of collagenous fibers is increased in β-aminopropionitrile-treated (lathyritic) rats (1), but their tensile strength is decreased (2), and below we show that this is true also per cross-sectional area of the fibers.

Heat activates the collagen molecules by breaking hydrogen bond cross links and thus allows the molecules to reach a state of larger entropy in shrunken form (3). A similar result is obtained with several chemical agents (4). It is customary to observe the T_s or hydrothermal shrinkage temperature, where the rate of contraction is so rapid that it seems abrupt, but some investigators have recorded also the shortening of the fiber plotted against time (5). Chvapil and Zahradnik (4) studied in detail the later, relaxation phase.

Most of the present experiments were made recording the isometric tension as a function of time, since some tension is always present, at least as the weight of the fiber, and it is accurate to measure. A similar method has also been used by Rigby (6). In the course of the work we found that the tension-time relationship, especially the relaxation phase, was more sensitive to many conditions and more informative than the T_s alone.

EXPERIMENTAL

Measurement of isometric tension.—Figure 1 shows the arrangements A and B for the measurement of the thermally induced tension, which was usually recorded at 6-sec. intervals, when the indicator was adjusted again to the balance mark, which was inspected with a magnifying glass. Thus the length of the fiber remained constant. In the first experiments with tube A the length of the fiber was 5 cm. on the average, varying about ± 20%. The yarn which connected the hook of the torsion balance and the fiber was Gun No. 50 yarn (J.&P. Coats), which was least elastic from several samples. In the second arrangement (B) no yarn was used, and the length of the fiber was standardized to 6.5 cm.

The temperature was controlled by an accuracy of 0.1 °C. The temperature in the inner tube (B) rose from the room temperature to the test temperature (56°–63 °C.) in about 1.5–2 minutes. It is recognized that under these circumstances the first portion of the curves was obtained under conditions of constantly changing temperature until the inner tube (B) reached the test temperature. As a consequence it was possible to detect fibers which possessed lowered shrinkage temperatures by their accelerated development of tension. Nevertheless, since the same rubber tubing and pump were used in all experiments, the results obtained with similar fibers were quite reproducible.

To illustrate the variation, the following means with the standard deviations are quoted for three sets of five curves (each set from fibers of the same tail): time to reach the maximal tension, 61.2 ± 7.8 sec., 63.6 ± 5.3 sec., and 62.4 ± 17.3 sec.; and the maximal tension, 107 ± 28 mg., 112 ± 31 mg., and 124 ± 82 mg. Most of the curves given in the figures are typical samples from groups of 2–4 curves. Usually only every second reading is indicated.

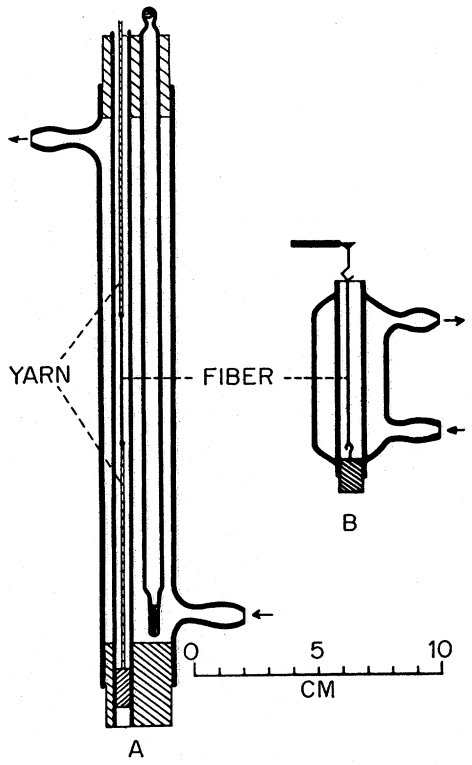

FIGURE 1.—Arrangements for the measurements. The upper end of the fiber (or the yarn) was connected in isometric measurement to the torsion balance (No. 2825, 5-mg. divisions, Vereenigde Draadfabrieken, Nijmegen, Holland) and in isotonic measurements to an indicating lever of the kymograph. The tube was attached to a telemicroscope stand in the place of the telescope. At the starting moment the pump was connected to circulate the water into the jacket from a constant-temperature bath. Usually a small weight (30–50 mg.) was put as a counterweight to "stabilize" the fiber, since otherwise the zero reading of the tension was not reached again. The initial reading of the balance was adjusted to zero, or sometimes to some positive value (about 30–50 mg.), by moving the whole measuring tube vertically with the fine screw of the telemicroscope.

Measurement of isotonic contraction.—Some experiments with tube B were made using a kymograph (Palmer, Electric 12, kindly put at our disposal by Dr. Niilo Kärki, then in Department of Pharmacology, University of Turku). The length ratio of the balanced arms was about 4:1 in favor of the tracing arm. Later the movements of the lever were recorded using an arched scale. This arrangement caused on the fiber a tension of about 12.5 mg.

Measurements of the tensile strength.—The fiber was immersed in water at room temperature (about 18°C.) and connected with a yarn to the beam of a balance. In the other side of the balance was a container, which

was filled with water with constant speed (about 250 ml/min) until the fiber broke. The water was weighed, and the force in grams per cross-sectional area of the fiber was calculated. For comparison some measurements were made at 4°C., but the results were the same as at room temperature.

Preparation of the rat tail tendons.—Rats of Wistar-strain were used —in most experiments animals designated as "adult", weighing about 180 g. The rat was killed with a blow on the head, and the fibers were extracted with forceps from a short wound which was made near the tip of the tail. Only the evenly thin "inner fibers" were used. The fibers which are near the surface taper to their ends and are not easily prepared, although they are coarser. The thickness of the inner fibers was about 0.15–0.20 mm. They were measured in the wet condition under a microscope, using an eyepiece with a graduated scale.

In the first experiments the lability of the fibers on storage was not realized, and the tails of the rats were kept frozen until the fibers were prepared immediately before use. The tensile strength was not affected by this storage. For the later studies of the properties of tension-time curve the fibers were used from rats immediately after killing.

Lathyritic rats.—Rats of 30–40 g. weight received aminoacetonitrile (Abbott Laboratories, Lot 1279–197, as hydrosulfate; gift of Dr. A. van den Hooff), 25–100 mg. daily mixed into the standard food of our laboratory. After 10–20 days the lathyritic symptoms appeared: retardation of weight gain, coarse fur, exostoses, and difficulty in movements. The rats were killed in pairs (a control and an experimental animal). In the earlier part of the work the tails were stored frozen 1–2 days, but later they were studied immediately.

RESULTS

Tensile strength in lathyrism.—The fibers from the lathyritic rats were generally thinner than the controls (average diameters 0.16 mm. and 0.19 mm., respectively). In both groups 18 fibers from 4 rats were measured. The average tensile strength in control samples was 760 g/mm^2, and in lathyritic samples it was 340 g/mm^2 ($P<0.01$).

The normal tension-time relationship.—Samples of the normal curve are seen in Figs. 2–10 as the control. We did not find any satisfactory condition for the storage. When the fibers were frozen and thawed, the maximal tension was appreciably decreased. Neither should the fibers be allowed to dry. The best medium is water at about 2°C., and the fibers could be kept about 1 hr. in water or in Ringer's solution at room temperature. The relaxation phase is accelerated after storage. Ringer's solution causes, in addition, a more rapid development of tension. The effects of storage in water at

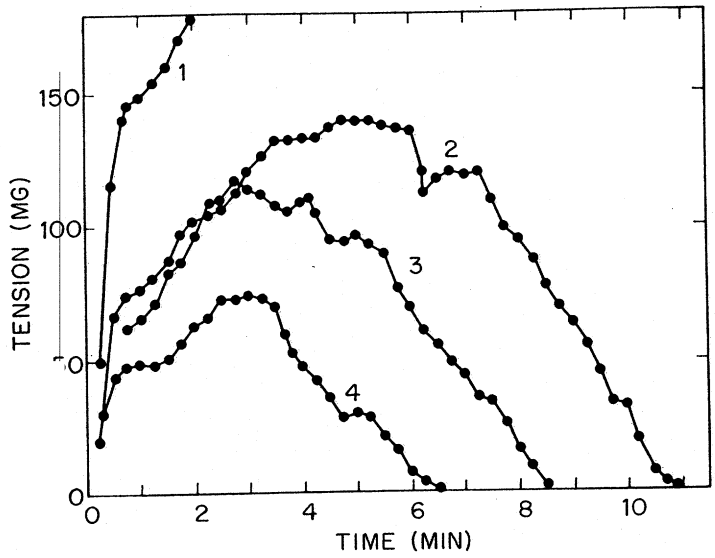

FIGURE 2.—Effect of storage in water at room temperature on the tension-time relationship. All measurements at 60°C. in water; stabilizing weight, 50 mg. Storage at room temperature in water before measurement: Curve *1*, 2 min.; curve *2*, 40 min.; curve *3*, 105 min.; curve *4*, 540 min. All fibers from the same rat, weight 175 g.

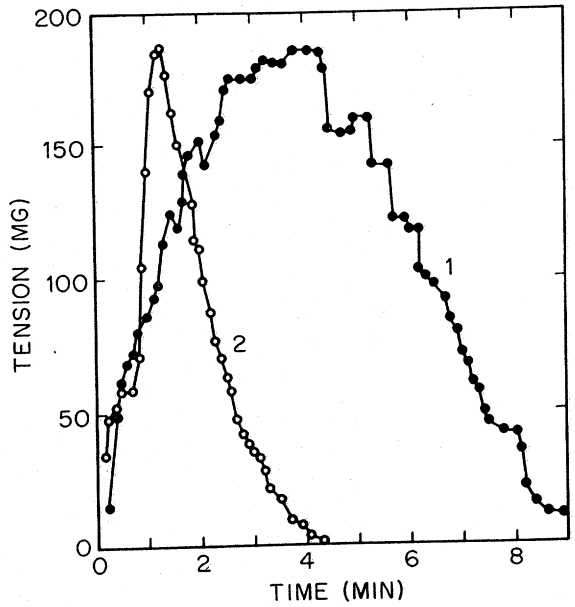

FIGURE 3.—Effect of storage in Ringer's solution (72 hr. at 4°C.) on the tension-time relationship. Measurements at 60°C. in water; stabilizing weight 50 mg. Curve *1* (●) presents the control; curve *2* (○), the experiment. All fibers from the same adult rat. Each curve represents a set of three.

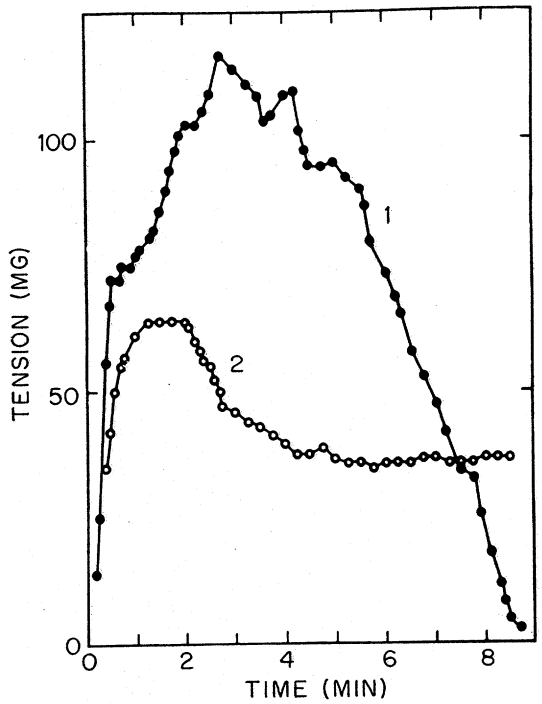

FIGURE 4.—The tension-time relationship in fibers from a young rat. All measurements at 60°C. in water; stabilizing weight 50 mg. Curve *1* (●) = fibers from a rat weighing 175 g. (about 100 days); curve *2* (○) = fibers from a rat weighing about 25 g. (age 20 days). Each curve represents a set of three.

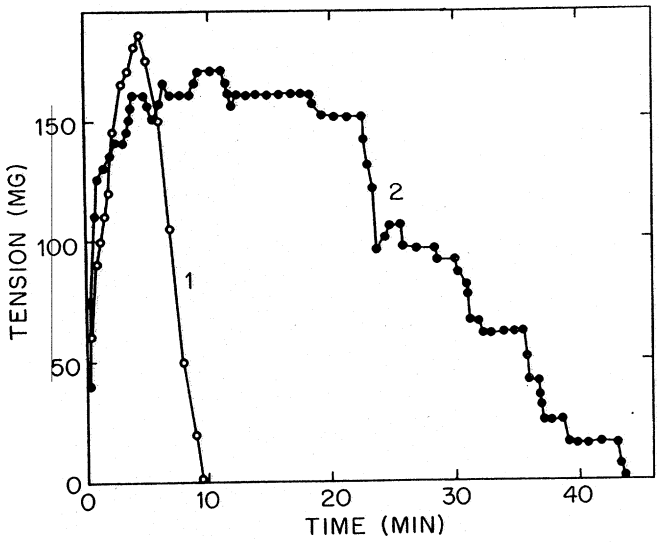

FIGURE 5.—Effect of temperature on the tension-time relationship. Curve *1* at +60°C.; curve *2* at +56°C.; both in water; stabilizing weight 50 mg. All fibers from the same adult rat. Each curve represents a set of two. (A measurement at 63°C. is given in Fig. 8).

room temperature and in Ringer's solution at 4°C. are presented in Figs. 2 and 3, respectively.

The fibers from young animals dissolved partly during the relaxation phase to a transparent gelatinous filament. Many times the fiber broke. However, the "residual tension" settled on a higher level if the fiber did not break (Fig. 4). No systematic study was made on the effect of the age of the animal or the thickness of the fiber, but the maximal tension seems to be larger the thicker the fibers, as they are in older rats. The relaxation phase depends on temperature (Fig. 5). In salt solutions the maximal tension is higher than in water (Fig. 6), and both phases are more rapid (cf. Fig. 3).

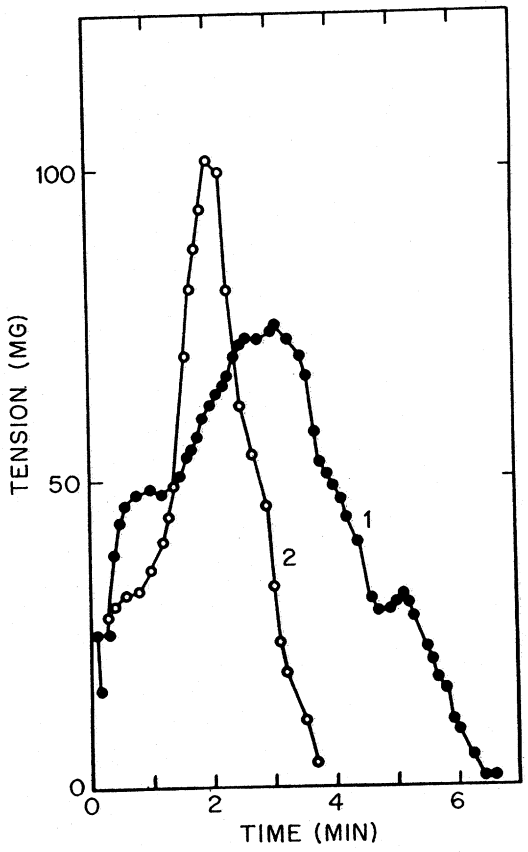

FIGURE 6.—Effect of the presence of NaCl on the tension-time relationship. Measurement at 60°C.; stabilizing weight 50 mg. Curve *1* (●) = control; curve *2* (○) = in 1*M* NaCl solution. Curves represent typical samples from two rats (175 g. and 250 g.), four from each. The fibers were measured immediately after the preparation.

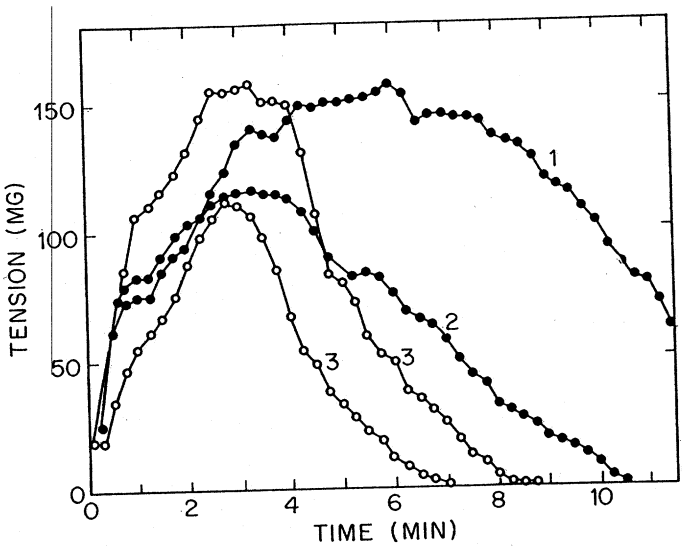

FIGURE 7.—Effect of pretreatment in 1*M* NaCl on the tension-time relationship. All measurements at 60°C. in water after a brief rinse; stabilizing weight 50 mg. Curve *1* (●) = 60 min., and curve 2 (●) = 210 min., in water at room temperature; curves *3* (○) = 150 min. in 1*M* NaCl (pH 7.4). All fibers were prepared at the same time from the same rat (175 g.) and kept in water or 1*M* NaCl at room temperature until measured. The curves represent sets of three. Lathyritic samples showed a similar behavior against pretreatment in NaCl solution.

Effect of pretreatments.—The pretreatments in 1*M* NaCl (Fig. 7), acetic acid (Fig. 8) and urea (Fig. 9) all accelerated the relaxation phase, and acetic acid caused, in addition, a more rapid development of tension. Also the immersion in NaCl solution caused an acceleration in the development of tension, which was already seen in Fig. 3 or in Fig. 6. It was thought that some of the pretreatments had caused a chemical contraction already at room temperature. This is proved by the demonstration of chemical contraction by acetic acid at temperatures below 10°C. (Fig. 10). The chemical contraction by urea was smaller.

In many occasions it was observed that the curve was not smoothly continuous (e.g., Fig. 11). Several attempts were made to clarify whether the "kinks" are regular. The results showed large variation, and the steps may occur at random and be due to the weak periods of the fiber, which were observed in the microscope. Before the development of the tension there is always a short latency period, with an initial relaxation phase, which is not explained. This is seen especially in curves of isotonic contraction (Fig. 13).

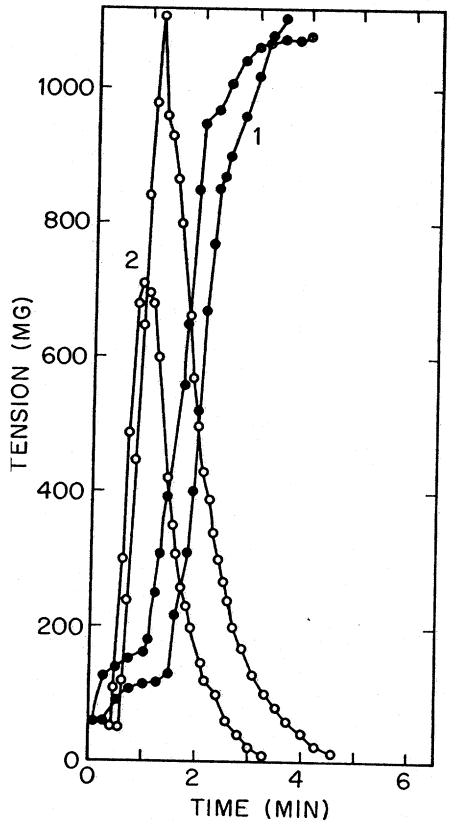

FIGURE 8.—Effect of pretreatment in acetic acid on the tension-time relationship. All measurements at 63°C. in water, after a brief rinse; stabilizing weight 50 mg. Initial reading 50 mg. Curves *1* (●) = controls; curves *2* (○) = after 10 min. at room temperature in 0.001*N* CH_3COOH. Fibers from the same rat. weight 270 g.

Effect of lathyrism.—The typical difference is shown in Fig. 11. The earlier occurrence of the maximal tension could be statistically verified ($P < 0.02$, 13 fibers from 4–5 rats in both groups). When the fibers had been stored for 3 days at 4°C. in Ringer's solution and studied at 60°C., the difference in the times necessary to reach the maximal tension persisted. The maximal tension decreased more in lathyritic samples.

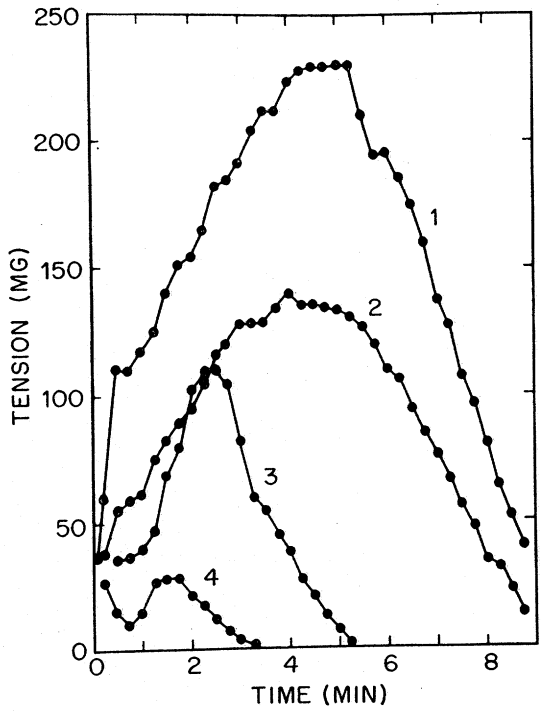

FIGURE 9.—Effect of pretreatment in urea on the tension-time relationship. All the measurements at 60°C. in water after a brief rinse; stabilizing weight 50 mg. Curve *1* = control, curve *2* = 5 min. in 4*M* urea, curve *3* = 8 min. in 4*N* urea, curve *4* = 12 min. in 4*N* urea at room temperature. All fibers prepared from the same adult rat.

Isotonic contraction.—The maximal tension and maximal contraction were reached simultaneously, as a rule. Figure 10 describes the chemical contraction of collagenous fibers at low temperature, measured also by the tension-time method. In this case large tension develops in spite of a still small contraction. To illustrate the rapid changes which happen in rat tail tendon and to corroborate the effects of storage on fibers (Fig. 2–3), the kymographic record of isotonic thermal contractions of consecutive fibers from the same rat is included in Fig. 12. The fiber relaxes more easily in the second and third experiment by the tension caused by the indicating lever. The effect of alternating temperatures is shown in Fig. 13. The thermally induced changes are all irreversible.

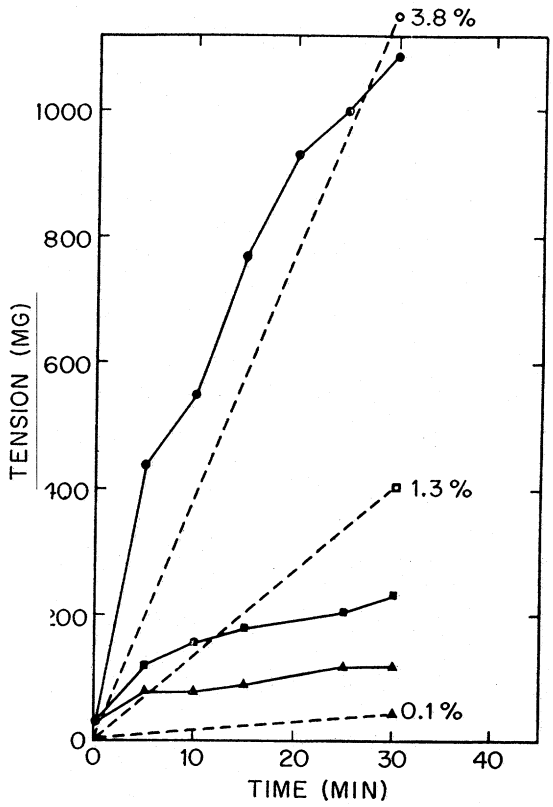

FIGURE 10.—Comparison of the chemical contraction by isometric and isotonic techniques. Measurements at 2°–10°C. (running tap water). Solid line and closed symbols indicate isometric tension, and dotted line and open symbols indicate isotonic contraction (figures give the percentage of shortening), ▲ △ in water, ■ □ in 1*M* urea, ● ○ in 0.001*N* CH_3COOH. Weight of rats 256–307 g. Stabilizing weight 30 mg.; initial reading 30 mg.

DISCUSSION

Form of the tension-time curve.—Figure 14 shows schematically the hypothetical changes in the collagen fiber. When the individual protofibrils contract but the total length of the fiber is maintained constant, they have to move in relation to each other. The force which opposes this movement and thus determines the rate of relaxation is the viscosity-like friction between the subunits. The form of the curve would not depend on the length or thickness of the fibril.

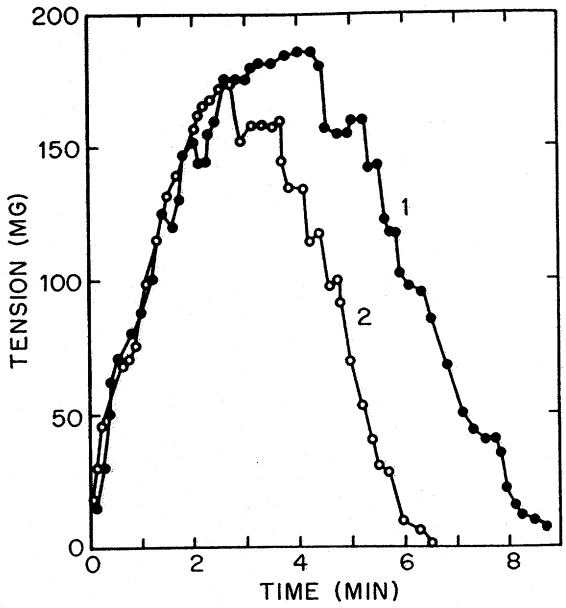

FIGURE 11.—Effect of lathyrism on the tension-time relationship. Measurements at 60°C. in water; stabilizing weight 50 mg. Curve *1* (●) = control; curve *2* (○) = fiber from a rat of the same age treated for 10 days with aminoacetonitrile (25 mg/day).

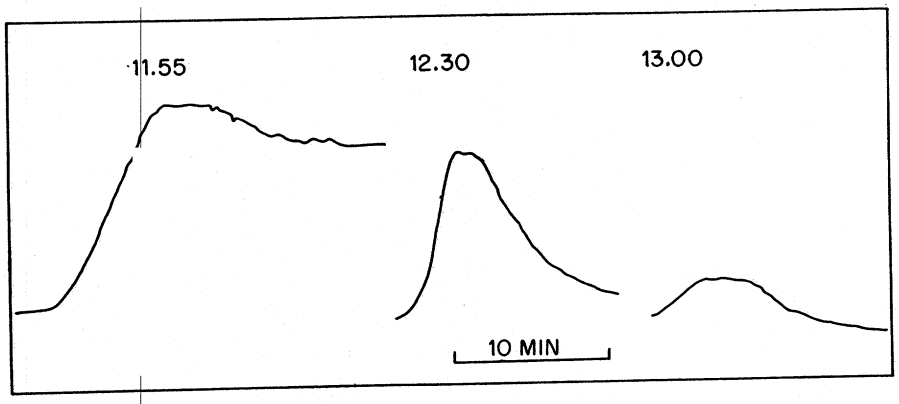

FIGURE 12.—Change of properties of rat tail tendon fibers extracted from the same tail at indicated times and studied by isotonic contraction (at 60°C.), as recorded with a kymograph. Weight of the rat, 307 g.; killed. about 11.50.

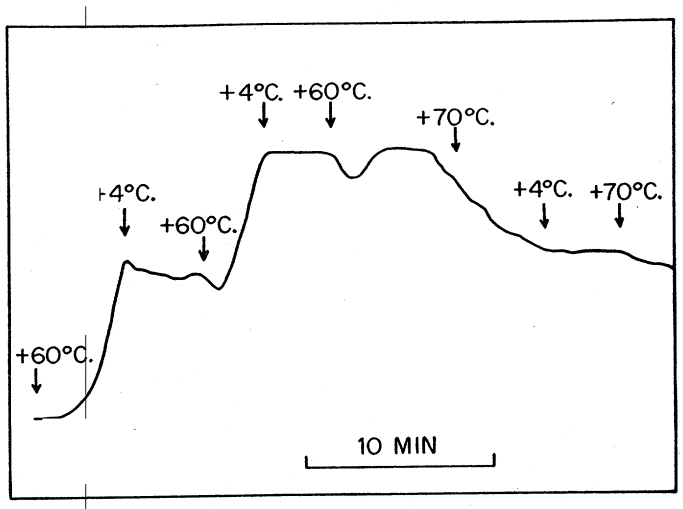

FIGURE 13.—Kymographic record of isotonic contraction of a fiber (length about 5 cm.) in water at alternating temperatures. Weight of rat, 254 g.

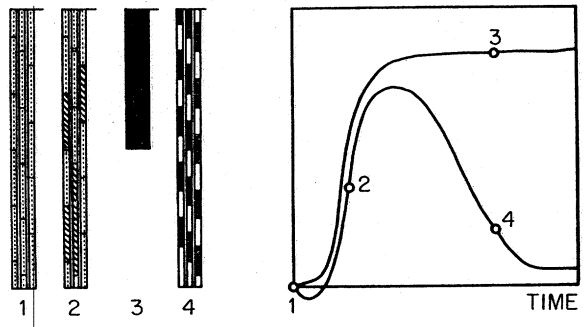

FIGURE 14.—Proposed schematical model on the effect of heat. *Right*: isotonic and isometric records plotted against time. Figures refer to the hypothetical situations in the fiber, *Left*: *1*: starting point, the macromolecules in the fiber are kept in extended positions by hydrogen bonds; *2*: isometric tension develops because of heat activation of the shadowed molecules at random; *3*: the end of isotonic contraction; (The fibrils have each contracted (in this example) to half the original length.) *4*: approaching the relaxed state of isometric tension. If all protofibrils contract to half their original length, but the original length of the fiber is to be maintained, the contracted units have to slide in relation to each other until "residual" tension is in balance with the frictional forces. The white "empty" spaces will be filled by the increased thickness of the contracted units.

It would be desirable to characterize the tension-time relationship with numerical parameters. An approximate expression for the similar curve of chemical contraction was derived by Chvapil and Zahradnik (4). We do not attempt the same but suggest the curve as a sum of two processes: (*a*) the collagen molecules become activated, as is evidenced in the development of tension (the forms of the contraction curves published by Weir [5] resemble the probability integral, which is to be expected if the activation happens at random.) and (*b*) the tension overcomes the frictional forces between the fibrils, the subunits shrink, and the tension begins to decrease. The observed tension is thus reduced by the work done to surmount the friction, and the tension at any given time during relaxation is equal to the friction.

The best characteristic for the rate of the activation phase would be the slope of the curve at some suitable point, for example, at half-maximum. The relaxation phase is characterized by (*a*) the tension at the start of the relaxation, (*b*) the time which the fiber holds before the onset of the relaxation, and (*c*) the rate of the relaxation or steepness of the decline of the tension.

The first phase, development of tension, seems identifiable with the thermal or chemical contraction. Substances which accelerate it, sodium chloride and acetic acid, are known as solubilizers of collagen. In lathyrism, where the collagen is unusually soluble, the rise of tension was not definitely more rapid than normal, but it has a tendency to be so (not clearly shown in Fig. 11).

Relaxation.—Wiederhorn, Reardon, and Browne (7) studied the stress-strain behavior of heat-shrunk tendon for an approximation of the segmental molecular weight of collagen between the cross links (for discussion, see Gustavson (3), p. 202–4 and Gustavson (8), p. 267–69). They assumed that the tension is inversely proportional to the segmental molecular weight and directly proportional to $(\alpha\text{-}1/\alpha_2)$, where α is the strain of the shrunken tendon. If by a given tension the strain increases (the rate of relaxation increases), the segmental molecular weight should also be increased and thus indicate diminished cross links, which represent one form of restraint against the movement of the contracted protofibrils.

The stress-relaxation at lower temperatures which was studied by Rigby, Hirai, Spikes, and Eyring (9) is reversible and, therefore, different from the relaxation discussed above.

The theory of viscosity can be applied: the friction between the fibrils depends, in addition to the viscosity coefficient of the surface layers of the fiber, on the distance between the surfaces of the insoluble cores of fibers and also on the area of their surfaces. Rapid relaxation indicates small frictional forces.

The frictional forces may be decreased and the relaxation accelerated by the following factors:

(*a*) Decrease of the viscosity of the soluble layer at the fiber surface. Gelatinization degrades the soluble collagens even below 60°C. (10). Fibers of the young rats are gelatinized almost totally. However, the adult fiber does not lose its gross appearance, and the gelatinization must occur only on the surfaces of the fibers. The viscosity of the heated form of soluble collagen on the surfaces of the fibers is much smaller than the viscosity of the original solution (11), and the movement of the fibrils is thus facilitated. The gelatinization explains the appearance of hydroxyproline in the immersion fluid during the relaxation, which was reported by Chvapil and Zahradnik (4). We can confirm this finding already at maximal tension point. Kessler, Rosen, and Levenson (12) found that after heating the rat tail tendons for 3 minutes at 65°C. the chromatographic pattern of the acid-solubilized collagen was altered. The effect of urea can also be explained by the gelatinization (11). It is also possible that in some conditions, e.g., in lathyrism, the collagen of adult rat gelatinizes more rapidly or that there are differences in the resulting gelatinous layer because of varying cross links.

(*b*) Loosening of the order and crystal packing of the collagenous protofibrils and increase in their distance. We believe that the effect of lathyrism is partly due to decreased orientation of molecules with loose crystal structure. Another factor which may decrease the cohesion between the fibrils is postmortem, or chemical, swelling. Its quantitative significance in the observed effect of storage, sodium chloride, and acetic acid remains to be investigated.

(*c*) Increased thickness of protofibrils with resulting relative decrease in moving surfaces and decreased frictional forces.

The effects of storage are obscure, but it seems relevant to mention the finding by Gross (13) that after storage at 3°C. for 48 hours the solubility of collagen in sodium chloride solution decreased rapidly. This may have happened either by physical transformation into insoluble form or by metabolic removal of the soluble form. The disappearance of a highly viscous layer of soluble collagen from the surfaces of the fibers would also decrease the friction. It is relevant to mention that in our hands storage at 37°C. was not better than at room temperature.

The relaxation rate and the tensile strength do not depend on identical, but only related, factors since the tensile strength, measured at room temperature, is not affected by storage (for discussion on cohesion and tensile strength, see Gustavson [3]).

ACKNOWLEDGMENT

We are indebted to Professor K. H. Gustavson, Stockholm, for criticism and encouragement. This work has been supported by a grant from the U. S. Department of Agriculture (P.L. 480).

REFERENCES

1. Levene, C. I., and Gross, J., *J. Exp. Med.*, **110**, 771 (1959); Mikkonen, L., Tuominen, T., and Kulonen, E., *Acta Chem. Scand.*, **13**, 2125 (1959).
2. Kalliomäki, L., Yli-Pohja, M., and Kulonen, E., *Experientia*, **13**, 495 (1957); Enzinger S. M., and Warner, E. D., *Lab. Investigation*, **6**, 251 (1957).
3. Gustavson, K. H. *The Chemistry and Reactivity of Collagen* (New York: Academic Press Inc., 1956), ch. 9.
4. Chvapil, M., and Zahradnik, R. *Biochim. Biophys. Acta*, **40**, 329 (1960).
5. Weir, C. E. *JALCA*, **44**, 108 (1949).
6. Rigby, B. J. *Biochim. Biophys. Acta*, **47**, 534 (1961).
7. Wiederhorn, N.W., Reardon, G. V., and Browne, A. R. *JALCA*, **48**, 7 (1953).
8. Gustavson, K. H. *The Chemistry of Tanning Processes*, (New York: Academic Press Inc., 1956), pp. 267–69.
9. Rigby, B. J., Hirai, N., Spikes, J. D., and Eyring, H. *J. Gen. Physiology*, **43**, 265 (1960).
10. Piez, K. A., Weiss, E., and Lewis, M. S., *J. Biol. Chem.*, **235**, 1987 (1960).
11. Mathews, M. B., Kulonen, E., and Dorfman, A. *Arch. Biochem. Biophysics*, **52**, 247 (1954).
12. Kessler, A., Rosen, H., and Levenson, S. M. *J. Biol. Chem.*, **235**, 989 (1960).
13. Gross, J. *J. Exp. Med.*, **107**, 247 (1958).